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Chemiluminescence immunoassay for chloramphenicol

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Abstract In recent years, various chemiluminescent clinical immunoassay kits have been widely applied to the detection of hormones. However, a kit for chloramphenicol (CAP) is often absent from most commercial product lists, even though it is important to control the levels of CAP residues in foodstuffs too. Therefore, we describe a simple, solid-phase chemiluminescence immunoassay (CLIA) for the measurement of CAP in foodstuffs. A rabbit anti-CAP IgG is passively adsorbed onto the walls of polypropylene plates. The labeled antigen is horseradish peroxidase (HRP) conjugate of CAP. Luminol solution is used as the substrate of HRP. The light yield is inversely proportional to the concentration of CAP. The method has a similar sensitivity (0.05 ng/ml), specificity, precision, and accuracy to a conventional enzyme immunoassay (EIA). The intra-assay and inter-assay CVs of ten samples were $<8\%$ and $<20\%$, respectively, and the analytical recovery of the method was 87–100%. The experimental correlation coefficient of dilution was found to be 0.999 using milk supernatant as buffer. The detection limit for the method was 0.1–10 ng/ml, and it displayed good linearity.

Keywords Chloramphenicol (CAP) · Chemiluminescence immunoassay (CLIA) · Horseradish peroxidase (HRP)

Introduction

Chemiluminescence (CL) is a highly sensitive method that enables non-isotopic detection in immunoassays [1, 2]. CL detection of molecules of synthetic or natural origin, such as proteins and nucleic acids (DNA and RNA), as well as other biological molecules, is increasingly replacing radioactive detection as the method of choice where sensitivity is critical. In many hospitals, CL immunoassay (CLIA) kits have become popular [3, 4, 5, 6]. They can detect most hormones and antibiotics with high sensitivity, specificity, precision, and accuracy. Horseradish peroxidase (HRP) is detected using one of the most popular CL substrates (H_2O_2 and luminol). A variety of substituted phenols have been known to act as enhancers for this system, such as 4-iodophenol [7], 4-phenylboronic acid [8] and 4-iodophenylboronic acid [9]. Toxicological problems associated with the administration of the drug chloramphenicol in humans are well documented [10]. The use of chloramphenicol is prohibited when breeding cattle and birds [11], because chloramphenicol (ingested via foodstuffs) is highly toxic to humans. In many countries, including China, a zero tolerance policy has been established for the presence of CAP residues in meat and animal products. CAP can be detected using various techniques, such as radioimmunoassay, enzyme immunoassay (EIA), high-performance liquid chromatography, and so on. Although EIA is one of the most widely used of these methods, many believe that it will be superceded by CLIA due to its superior performance. We have therefore investigated a CAP detection system based on CL immunoassay, and using HRP as the enzyme label.

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Materials and methods

Apparatus and reagents

A luminometer (Victor^2 1420 Multilabel Counter) with software was purchased from Wallac (Gaithersburg, MD, USA). EIA-Micro and CLIA-Micro 96-well microtiter plates were purchased from NUNC (Denmark). Chloramphenicol (CAP) and rabbit anti-CAP IgG were supplied by Beijing Atom Hightech Co. Ltd. Horseradish peroxidase (HRP), dicyclohexyl carbodiimide (DCC), *N*-hydroxysuccinimide ester (NHS), bovine ser-

um albumin (BSA) and *N*-ethyl-*N*′-[3-(dimethyl)aminopropyl] carbodiimide hydrochloride (EDC) were all purchased from Sigma (St. Louis, USA).

Assay protocol

EIA protocol (used to check the stabilities of the standards, the CAP-labeled, coated plates, and other prophase work): Rabbit anti-CAP IgG were diluted in 0.05 M, pH 9.5 carbonate buffer (CB) to particular concentrations, and then used to coat the EIA-microtiter plates and incubated overnight at 4 °C. Before the experiment, the plate was washed with 0.02 M, pH 7.4 phosphate buffer (PB), and unoccupied sites were blocked with the blocking solution (1% BSA) for 1 h at 37 °C. The plate was washed, and then labeled conjugates and standards diluted to appropriate concentrations in diluent were added. Upon every dilution, the absorbance was measured at 450 nm (OD_{450}).

CLIA protocol (used to review assay performance and results): immunoreactions were performed as described for the EIA protocol, except that the luminescence of each well was checked after adding CL substrate too. These luminescence results are given in counts per second (cps).

Purification of rabbit anti-CAP IgG

About 1 ml of rabbit anti-CAP serum was purified with Hitrap Protein G HP. The concentration of rabbit anti-CAP IgG was 0.815 mg/ml, shown by the UV absorption at 280 nm.

Synthesis of HRP-conjugated CAP

The CAP (1 mg), NHS (1 mg) and DCC (1 mg) were mixed and stirred in 0.1 ml dimethylformamide (DMF) for 4 h at room temperature. The horseradish peroxidase (HRP, 1.0 mg) in 0.5 ml of 0.2 M $NaHCO_3$ was then added to the reaction mixture with stirring. After the addition of EDC (1 mg), the reaction mixture was stirred at room temperature overnight. The reaction solutions were then dialyzed against PBS for two days, then mixed with 2 ml glycerol and stored at −20 °C [12, 13].

Results

Selecting the dilution level of labeled CAP

The EIA-microtiter plates were coated with rabbit anti-CAP IgG (1.5 μg/ml). Labeled conjugates diluted to appropriate concentrations in diluent were added. Upon every dilution we monitored the OD_{450} counts for standard S_0 (the CAP concentration was 0 ng/ml) and NSB (nonspecific binding). 100 μl of standard CAP and 100 μl of labeled CAP were added to each well. The results are shown in Table 1. Based on these results, we chose a dilution level of 1:10000 for the labeled CAP.

Table 1 Selecting the dilution level of labeled CAP

OD_{450} counts for...	1:2000	1:4000	1:8000	1:16000	1:24000	1:48000
NSB	0.115	0.097	0.089	0.076	0.081	0.090
S_0	3.107	3.090	2.541	2.233	1.833	1.587

Selecting the concentration of the antibody used for the coating

The antibody was diluted to 2, 3, 4, 5 μl in order to coat the EIA-microtiter plates for immunoreaction with the labeled CAP diluted 1:10000, and the results (in terms of OD_{450} counts) are shown in Table 2.

From the results shown in Table 2, we considered that 3 μg/ml was the appropriate concentration of antibody to use in the following reaction.

Selecting the incubation period

We diluted the standards to 0, 0.1, 30 and 100 ng/ml for immunoreaction. The plates with the standards and the label were incubated for 0.5, 1, 2, and 4 h, then we reviewed the changes in the OD_{450} counts. The results are shown in Fig. 1 (count in OD_{450}).

According to the results shown and Fig. 1, it takes nearly 3 h to reach reaction equilibrium, so an incubation time of 1 h is acceptable.

Table 2 Selecting the concentration of the antibody used for the coating (values given are the OD_{450} counts)

Standard	2 μg/ml	3 μg/ml	4 μg/ml	5 μg/ml
0	2.101	2.281	2.107	2.519
0.1 ng/ml	1.845	2.045	1.871	2.204
30 ng/ml	0.202	0.217	0.200	0.263
100 ng/ml	0.119	0.125	0.117	0.147

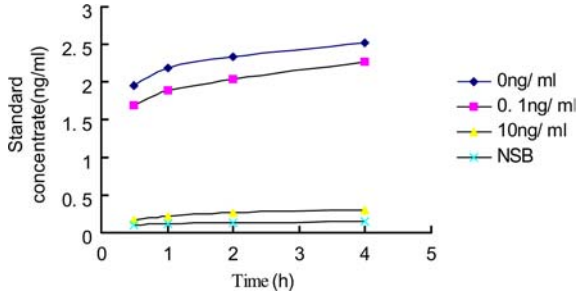

Fig. 1 Selecting the incubation period

The stability of labeled CAP

The labeled CAP was diluted to 1:100, and its stability (in terms of OD_{450} counts) was monitored for eight days at 37 °C. The results are shown in Fig. 2.

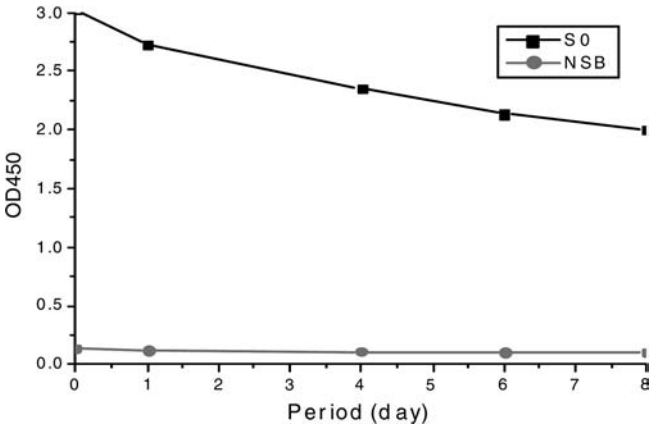

Fig. 2 The stability of labeled CAP at 37 °C

Stabilities of the standards

The standards were diluted to 1 and 10 ng/ml (low and high concentrations, respectively), and their stabilities were monitored at various temperatures over 14 days. The results are shown in Fig. 3.

According to Fig. 3, the standards were most stable at 4 °C.

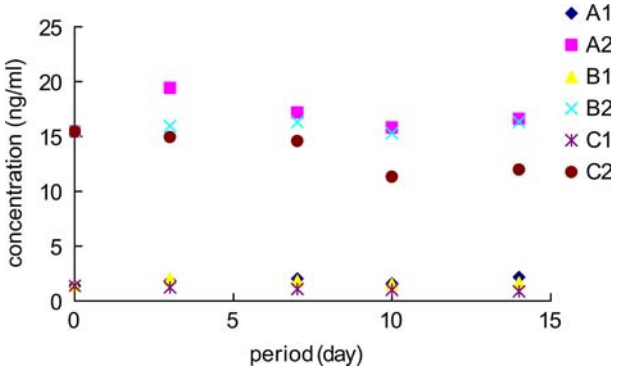

Fig. 3 The stabilities of the standards over 14 days. *A1* standard at low concentration at 4 °C, *A2* standard at high concentration at 4 °C, *B1* standard at low concentration at room temperature, *B2* standard at high concentration at room temperatures, *C1* standard at low concentration at 37 °C, *C2* standard at high concentration at 37 °C

Stability of the coated plates

Plates were coated in the antibody at a concentration of 3 μg/ml, and the stabilities (in terms of OD_{450} counts) of the coated plates were monitored for ten days at 37 °C. The results are shown in Fig. 4.

The luminescence persistence of the CL substrate

After immunoreaction, 180 μl of CLIA substrate was added to each well, and then the luminescence was monitored every 15 min. The luminescence results are shown in Table 3.

We can obtain the concentrations of the samples by constructing a luminescence vs concentration calibration curve. Now, it takes a significant time, 52 s, for all of the samples to be measured during one detection cycle, and since the luminescence is time-dependent, we must account for the fact that the samples are not actually sampled simultaneously during each cylce. Therefore, in Table 4, each row represents the results for a particular detection cycle, "I" shows the concentrations of the samples obtained via the standard calibration curve, "II" shows the concentrations of the samples obtained via the standard calibration curve at the end of the detection cycle, and "III" shows the relative error: III = (II − I)/I×100.

According to the upper data we consider that offset error is overlook, and we can get perfect result after adding our CLIA substrate in 3–15 min.

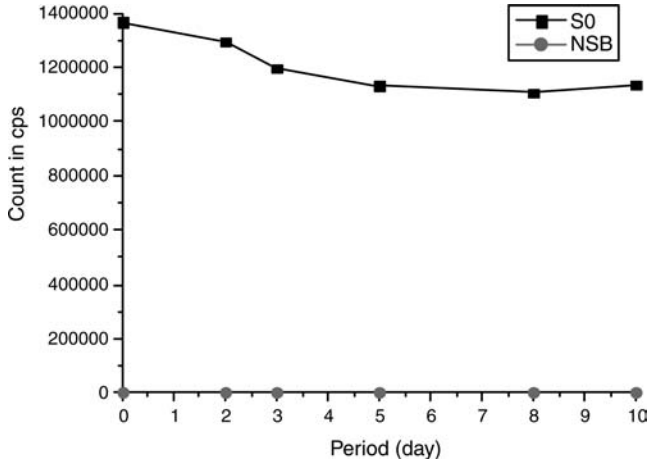

Fig. 4 Stability of the coated plates

The linearity of the calibration curve and the limit of quantitation

We diluted the standards to 0, 0.1, 0.3, 1, 3 and 10 ng/ml, then 100 μl of standard and 50 μl of label was added to wells coated in antibody for immunoreaction. We monitored the counts per second from each well, and constructed a luminescence vs concentration calibration curve (see Fig. 5). According to the curve we can reliably quantitate the levels of CAP in foodstuff samples from

Table 3 Luminescence results (in counts per second) for all standards (S) and samples (Sm)

	S_0 (cps)	S_1 (cps)	S_2 (cps)	S_3 (cps)	S_4 (cps)	S_5 (cps)	NSB (cps)	Sm_1 (cps)	Sm_2 (cps)	Sm_3 (cps)
1 time	1100675	960585	889015	721455	390660	156010	10220	812170	442475	214015
2 time	1160845	1012010	941185	759515	431455	180100	15040	840685	476425	239970
3 time	1170565	1012865	955660	768500	444090	194910	18550	847040	484560	253040
4 time	1166125	1013730	955975	768625	450000	200685	20140	847390	492185	258850
5 time	1153200	1004670	950185	766725	449465	206360	21820	841855	488300	260210
6 time	1143440	999065	943430	762095	449620	205965	23280	840480	488700	260025
7 time	1128535	987570	936395	760000	449100	205515	23460	833190	486435	262540
8 time	1114510	977635	923985	750290	441555	204865	24250	824805	487955	263110
9 time	1098250	959700	917390	743695	442785	205040	25510	816875	48290	261725
10 time	1072685	953570	904460	736325	440050	204765	23340	807315	479635	261180
11 time	1062680	933010	891770	719140	434020	199940	23670	790930	471325	257350
12 time	1043050	917115	878630	713815	430000	198725	23930	782730	467490	259595
13 time	1031005	904830	862765	704080	423560	195430	23550	772075	460790	256355
14 time	1008200	888605	851210	696225	423655	197195	23920	760880	458620	254075
15 time	994680	880340	837205	686150	416200	193930	23110	750800	452285	254000
16 time	978140	860750	829565	676355	410140	193405	22890	737445	445510	250475

Table 4 The samples' offset and unoffset and relative error by 16 times

	I			II			III		
	Sm1 (ng/ml)	Sm2 (ng/ml)	Sm3 (ng/ml)	Sm1 (ng/ml)	Sm2 (ng/ml)	Sm3 (ng/ml)	Sm1 (%)	Sm2 (%)	Sm3 (%)
1 time	0.132	0.897	2.932	–	–	–	–	–	–
2 time	0.145	0.888	2.873	0.114	0.753	2.563	21	15.2	10.8
3 time	0.141	0.853	2.692	0.141	0.853	2.692	4.1	4.5	4.7
4 time	0.147	0.860	2.744	0.147	0.860	2.744	0.6	1.3	1.7
5 time	0.152	0.889	2.800	0.152	0.889	2.773	2.0	0.5	1.0
6 time	0.147	0.874	2.800	0.150	0.883	2.803	2.7	1.0	0.1
7 time	0.147	0.872	2.758	0.151	0.885	2.764	2.7	1.5	0.2
8 time	0.145	0.840	2.710	0.153	0.865	2.750	5.5	3.0	1.5
9 time	0.144	0.846	2.731	0.151	0.863	2.730	4.9	2.0	0.0
10 time	0.146	0.840	2.700	0.152	0.861	2.738	4.1	2.5	1.4
11 time	0.147	0.837	2.688	0.159	0.878	2.756	8.2	4.9	2.5
12 time	0.145	0.830	2.626	0.153	0.855	2.655	5.5	3.0	1.1
13 time	0.144	0.828	2.615	0.153	0.861	2.674	5.5	4.0	2.2
14 time	0.144	0.817	2.641	0.153	0.838	2.649	6.3	2.6	0.3
15 time	0.144	0.815	2.577	0.153	0.847	2.643	6.3	3.9	2.6
16 time	0.146	0.817	2.589	0.156	0.848	2.630	6.8	3.8	1.6

10 ng/ml to 0.1 ng/ml with good linearity. This method can be used to detect CAP concentrations as low as 0.1 ng/ml.

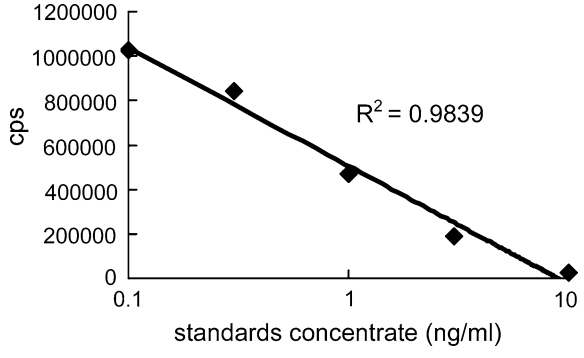

Fig. 5 The luminescence vs concentration calibration curve

The sensitivity of the method

100 µl of standard and 50 µl of labeled CAP were added to wells coated in antibody (200 µl at 3 µg/ml) and left overnight. After immunoreaction and washing-up, we added 180 µl of CLIA substrate to each well and then detected the luminescence after five minutes. Ten samples were monitored in this experiment. After reviewing the data, we calculated the sensitivity of the method to be 0.046 ng/ml.

The precision of the method

The luminescence of three different samples were monitored over 30 days, and the intra-assay and inter-assay CVs were derived. These are shown in Table 5.

From the results in Table 5, we can see that the intra-assay CVs were $<8\%$, and the inter-assay CVs were $<20\%$.

Table 5 Determining the precision

Samples	Intra-assay CVs ($n = 10$)			Inter-assay CVs ($n = 10$)		
	(ng/ml)	s (ng/ml)	CV (%)	(ng/ml)	s (ng/ml)	CV (%)
Light	0.189	0.014	7.7	0.130	0.025	19.6
Medium	0.807	0.064	7.9	0.739	0.086	11.6
Heavy	2.395	0.096	4.0	2.367	0.149	6.2

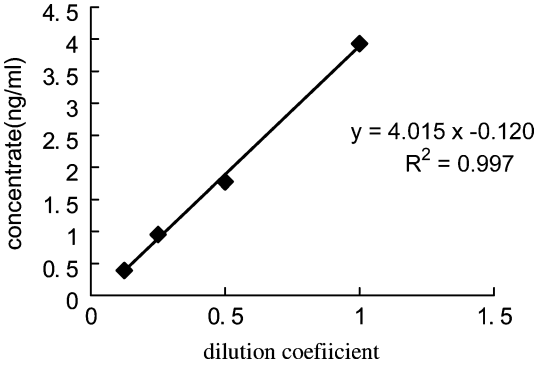

Fig. 6 The correlation coefficient of dilution experiment

The correlation coefficient of dilution experiment

We pretreated skim milk with Carrez reagents [14], and diluted the supernatant with standard diluent 1:1. We regarded the upper solution as the sample diluent, and diluted the sample to 1/2, 1/4, 1/8. The results are shown in Fig. 6.

The analytical recovery of CAP from the skim milk sample

We added 1, 4 and 10 ng of CAP to samples of 5 ml of skim milk, and then pretreated the milk as in the dilution experiment. We then derived the concentration of CAP in the skim milk using our developed method. The results are shown in Table 6.

From the data in the table, we can see that the analytical recovery of CAP can be acceptable.

Discussion

The label is not stable at room temperature or at 37 °C, but at 4 °C it is very stable, for at least a few months.

Table 6 The analytical recovery of CAP from the skimmed milk sample

Fortified concentration of CAP (ng/ml)	Measured concentration of CAP (ng/ml)	Recovery of CAP (%)
0.196	0.166	87.4
0.741	0.650	87.7
1.96	1.97	100.6

From these facts, we conclude that HRP-conjugated CAP is different to HRP-conjugated protein. CAP is a small molecule and it conjugates with HRP via a single chemical bond. As the temperature rises, this bond has a greater tendency to break.

The concentrations of standards used ranged from 0.1 ng/ml to 10 ng/ml. However, this does not mean that the technique is only linear over this concentration range; in fact, if the results for a 0.05 ng/ml standard are added to the calibration curve, the range 0.05 ng/ml to 10 ng/ml still has good linearity.

Because the sensitivity [15] (0.05 ng/ml) with 3μg/ml antibody well coating is acceptable, We recommend an antibody concentration of 3 mg/ml in order to improve the sensitivity and the linearity range for special users. If necessary, however, we can make the sensitivity almost 10 ppt, or lower.

Although the immunoreaction reaches equilibrium after 3 h of incubation, the experiment shows that acceptable results can be achieved after 1 h incubation. In most cases, especially in hospitals, it is imperative to obtain experiment data as quickly as possible, so 1 h incubation is reasonable.

Compared to the results from ELISA, CLIA has a more cragged standard curve, which is why it has a higher sensitivity and a wider detection limit. Otherwise, the CLIA has more effective numeric than ELISA, so CLIA has a higher precision. According to the intra-and inter-assay CVs, we can know about the above conclusion.

It is important to obtain an idea of the persistence of the substrate luminescence over time [16, 17]. It will result in a larger error if the persistence is not as good, because the apparatus detects the luminescence well by well, so the luminescence offset error (caused by the finite time, 52 s, taken to measure all of the wells in sequence) becomes more important. We have proved that, after 3–15 min of the onset of luminescence, the relative luminescence offset error within each set of well measurements is $< 5\%$. If the samples are monitored every 5 min, we can reduce the luminescence offset error to $< 3\%$.

Aside from the skim milk sample, we determined the CAP in some other foodstuff samples [18, 19]. The recoveries are also satisfactory, and the correlation coefficients in the dilution experiments are > 0.99. Our CAP-CLIA technique is therefore suitable for detecting CAP in various foodstuff samples.

Conflict of interest: No information supplied

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